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(54) Title: PORCINE CELLS INCAPABLE OF EXPRESSING CD40 ANTIGEN, FOR XENOTRANSPLANTATION

(57) Abstract

Provided are graftable mammal, preferably porcine, cells, tissues or organs comprising cells which are genetically modified to render them incapable of expressing CD40 antigen, and their use to prevent or inhibit chronic xenograft rejection in a recipient mammal receiving a xenograft.

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PORCINE CELLS INCAPABLE OF EXPRESSING CD40 ANTIGEN, FOR XENOTRANSPLANTATION

The present invention relates to biologically compatible material for use in transplants and to the production and use of such material.

The replacement of dysfunctional animal (particularly human) tissue, including organs, has over the last four decades become a common place therapy in clinical management. Organ transplantation has undergone significant development with modern immunosuppressants and the demand for organ transplantation has increased rapidly. Conventional transplantation surgery involves the transplantation from one animal of a particular species (generally human) to another of the same species. Such transplantations are termed allografts. Because of the current shortage of human donors for transplantable allografts, attention has focused on the possibility of using xenografts (transplants between species) in transplantation.

Spectacular progresses have recently been made in xenotransplantation and the pig stands out as the leading donor animal in the scientific and medical communities. It is believed that one of the major obstacles in transplanting successfully pig xenografts in humans is immunological, due to the presence of naturally occurring antibodies which leads to hyperacute rejection. A further obstacle is the chronic rejection in which the response of the blood vessel wall in the grafted organ to injury ("response-to-injury" reaction) plays an important role, thus leading to deleterious vasculopathies. Moreover, for long term survival and function, additional strategies must be devised to overcome the other immunological barriers of cell-mediated rejection.

Direct antigen presentation and high T cell precursor frequencies are thought to account for the speed and strength of acute allograft rejection. The nature of the human cellular response to porcine tissues is being investigated and results are presented below. It has been shown that human T cells can directly recognise pig SLA, and that the human T cell precursor frequency for SLA is higher than for HLA. T cell activation requires both engagement of the TCR by peptide/MHC and costimulation via one or more accessory molecules. A number of porcine accessory molecules have been shown to be compatible

with their human ligands including CD86, CD58 (LFA-3), and CD54 (ICAM-1). Therefore, it is likely that human T cells are capable of mounting a direct response to a pig xenograft.

It is now becoming clear that the role of CD40 is central to T cell activation, through the regulation of the expression of accessory molecules. Ligation of CD40 leads to up-regulation of accessory molecules in a number of cell types including B cells, endothelial cells and dendritic cells. There is also evidence to suggest that cross linking of CD154 to the CD40 receptor provides a direct stimulus to the T cell. This may be a critical step in the initiation of cell mediated responses.

Blocking antibodies to CD154 have been shown to prolong cardiac and pancreatic islet allografts in mice. Simultaneous blockade of both CD40 and CD28 pathways has been shown to further enhance cardiac and skin allograft survival in the mouse, and renal allograft survival in primates. Furthermore, this approach can greatly enhance survival of rat and pig skin grafts in mice.

It is therefore desirable to provide modified animal tissues and organs for xenotransplantation into a recipient in need of such tissue or organ transplantation with a significantly decreased risk of graft rejection due to cell mediated effects.

According to a first aspect of the invention, there is provided:

1.1 A method of preventing or inhibiting cellular graft rejection in a recipient mammal receiving a xenograft comprising using CD40-deficient donor mammal cells, tissues or organs as xenograft.

1.2 A method of preventing or inhibiting cellular graft rejection in a recipient mammal receiving a xenograft comprising isolating cells, tissues or organs to be transplanted from a CD40-deficient donor mammal and transplanting said CD40-deficient cells, tissues or organs into the recipient mammal.

The donor mammal may be a mouse, monkey, ape, pig, goat or cow, preferably a pig.

According to a further aspect of the invention, there is provided:

2.1 Graftable mammal, preferably porcine, cells, tissues or organs, particularly such comprising CD40-deficient porcine EC, which are genetically modified to render them incapable of expressing CD40 antigen.

The genetic modification may be an inactivation of the gene encoding CD40 antigen or an insertion of antisense RNA interfering with CD40 antigen expression.

The genetically modified cells, tissues or organs are useful e.g. in preventing in humans symptoms associated with organ or tissue xenograft transplant rejection, e.g. heart, lung, combined heart-lung, liver, kidney, pancreatic (complete or partial, e.g. Langerhans islets), skin, corneal transplants or bone marrow, particularly transplant vasculopathies.

According to a further aspect of the invention, there is provided a method of transplanting CD40-deficient porcine cells, tissues or organs into a recipient, the method comprising grafting the CD40-deficient porcine cells, tissues or organs in the recipient. Preferably the pig CD40-deficient cells, tissues or organs comprise CD40-deficient EC.

The present invention also comprises the porcine nucleic acid sequence that encodes a porcine polypeptide having CD40 antigen activity. Variations on this sequence that may be routinely generated by the skilled person include those varying within the scope of the degeneracy of the genetic code. The present invention also includes a porcine nucleic acid sequence that encodes porcine CD40 antigen and that hybridizes under standard high stringency conditions with a sequence complementary within the scope of the degeneracy of the genetic code. The complementary strands to above nucleic acid sequences are determined by standard methods and are also within the scope of the invention.

The present invention also comprises a host cell transformed with any of above mentioned nucleic acid molecules, as well as porcine CD40 antigen encoded by such transforming nucleic acid molecules and expressed from the host cell. Methods for transforming appropriate host cells and for expressing polypeptides from such host cells are known in the art and are described, for example, in Sambrook et al., (1984), 12.2-12.44; 16.3-17.44.

The invention further comprises a DNA construct useful for inactivating the gene encoding porcine CD40 antigen by insertion of a desired DNA sequence into an insertion site of said gene. Said gene includes the exons encoding or potentially encoding CD40antigen, introns contigous with such exons and regulatory elements associated with such exons and introns. The DNA construct includes the desired DNA sequence flanked by first and second homology sequences. These first and second homology sequences are sufficiently homologous, respectively, to first and second genomic sequences flanking the insertion site to allow for homologous recombination of the DNA construct with the porcine gene encoding CD40 antigen when the DNA construct is introduced into a target cell containing the porcine CD40 antigen gene.

A replacement-type DNA construct containing part of the genomic CD40 antigen gene sequence with a mutant foreign sequence such as a positive selection marker inserted within the coding region, may also be used instead of an insertion construct for inactivating the gene encoding the CD40 antigen. The construct is linearized outside the region of homology. Such a construct recombines with the CD40 antigen gene by a double cross-over event, resulting in the replacement of the chromosomal DNA.

To generate constructs for inactivating genes by homologous recombination, the gene is preferably interrupted within an appropriate coding exon by insertion of an additional DNA fragment, preferably a gene coding for a selectable marker. Specific exons may be identified as preferred locations for disruption of the gene by homologous recombination. The regulatory elements associated with the coding sequence may also present useful targets for inactivation.

In a preferred embodiment for inactivation of the gene encoding the CD40 antigen, a SalI site may be engineered into the corresponding location of the pig sequence for convenient construction of a knock-out sequence. The presence of a SalI site in the exon is useful since it is not likely that other SalI sites will be present at other locations in the knock-out constructs.

Preferably, the selectable marker is flanked by sequences homologous to the sequences flanking the desired insertion site. Any selective marker suitable for inclusion in a knock-out

vector may be used. The gene imparting resistance to the antibiotic G418 (neomycin derivative) frequently is used although other antibiotic resistance markers (e.g. hygromycin) also may be employed. Other selection systems include negative selection markers, e.g. the thymidine kinase gene from Herpes simplex.

The invention also includes methods for generating a pig totipotent cell having at least one inactivated (non-functional) CD40 antigen allele. By totipotent cells is understood pluripotent cells (cells capable of giving rise to at least several differentiated cell types) capable of giving rise to all cell types of an embryo, including germ cells. The totipotent cells can include without limitation embryonic stem (ES) cells, e.g. blastocysts, primordial germ cells (PG) and eggs. A "functional" allele is capable of being transcribed and translated to produce a polypeptide having an activity the same as or substantially similar to the native CD40 antigen. The methods include providing a plurality of cells characterized as pig totipotent cells, introducing into the totipotent cells a nucleic acid construct effective for inactivating the CD40 antigen gene by insertion of a desired DNA sequence into an insertion site of the gene through homologous recombination, and then identifying a totipotent cell having at least one inactivated CD40 antigen allele.

Due to the relative infrequency of homologous recombination in targeted cells, most such cells will carry only one inactivated allele of the target gene. The great majority of cells taken through a single round of transformation with an appropriate knock-out construct will be heterozygotes. The term "transformation" is defined as introduction of exogenous DNA into the target cell by any means known; e.g. transfection, microinjection, infection (e.g. with a retroviral vector), electroporation and microballistics. Although heterozygous cells can be used in the methods of the present invention, various manipulations can be employed to generate homozygous cells in culture. For example, homozygous cells can be generated by performing a second homologous recombination procedure on cells heterozygous for the CD40 antigen inactivated allele. If the knock-out construct used in the initial transformation carries one specific selectable marker, a second construct may be employed in a second round of transformation in which the selectable marker is replaced with a different selectable marker. Cells resistant to both selectable markers can be screened by Southern blots in order to detect any "double knock-outs" (i.e. homozygotes).

The invention further includes methods for generating a pig lacking a gene encoding functional CD40 antigen, preferably a transgenic pig with CD40-deficient EC. The methods include providing a pig totipotent cell having at least one inactivated CD40 antigen allele, manipulating the totipotent cell such that mitotic descendants of the cell constitute all or part of a developing embryo, allowing the embryo to develop to term, recovering a neonate individual derived from the embryo and raising and breeding the neonate to obtain a pig homozygous for an inactivated CD40 antigen allele, i.e. in which both CD40 antigen alleles are inactivated.

ES and PG cells are manipulated in various ways such that their mitotic descendants are found in a developing embryo. these manipulations can include, without limitation, injection into a blastocyst or morula, co-culture with a zona pellucida-disrupted morula, and fusion with an enucleated zygote. Cells injected into a blastocyst- or morula-stage embryo become incorporated into the inner cell mass of the blastocyst embryo, giving rise to various differentiated cell types of the resulting embryo, including in some cases germ cells. The embryo derived from such manipulations is a chimera composed of normal embryonic ES or PG cells. Alternatively, chimeric embryos can be obtained by co-culturing at least one ES or PC cell with a morula embryo in which the zona pellucida is sufficiently disrupted to allow direct contact between ES cell/PC cell and at least one cell of the morula. The zona pellucida-disrupted embryo may be an embryo that is completely free of the zona pellucida. Finally, the genome of an ES or PG cell can be incorporated into an embryo by fusing the ES cell/PG cell with an enucleated zygote. Such a procedure is capable of generating a non-chimeric embryo, i.e. an embryo in which all nuclei are mitotic descendants of the fused ES cell/PG cell nucleus. The resulting embryos are implanted in a recipient female or surrogate mother, allowed to develop to term, raised to sexual maturity and bred to obtain pigs carrying the inactivated CD40 antigen allele. If the original ES cell was heterozygous for the inactivated CD40 antigen allele, several of these animals will be bred with each other to generate animals homozygous for the inactivated CD40 antigen gene.

When eggs, as opposed to ES or PG cells, are directly injected with a nucleic acid construct effective for inactivating the CD40 antigen gene, the eggs can be manipulated to form and embryo by implanting into a recipient female. The direct injection of eggs can be a useful approach since it avoids the special manipulations required to turn a cultured cell into an animal. Fertilized eggs are "totipotent", i.e. capable of developing into an adult without

further substantive manipulation other than implantation into a surrogate mother. It may be useful to prepare the knock-out constructs from isogenic DNA, e.g. the constructs may be prepared from DNA isolated from the boar whose sperm is employed to fertilize the eggs used for injection.

Embryos derived from microinjected eggs can be screened for homologous recombination events in several ways, e.g. using the detection properties of the detectable marker inserted in the CD40 antigen gene. Alternatively the piglets may be analyzed by polymerase chain reaction (PCR) or reverse transcription PCR for evidence of homologous recombination.

Transgenic CD40-deficient pig tissue or cells may also be prepared by causing the expression of antisense RNA molecules interfering with the expression of CD40 antigen, particularly in EC. Exogenous nucleic acid molecules designed to specifically inhibit the expression of the native CD40 antigen within a pig cell may be used. These genetic manipulations include the use of nucleic acid molecules specially engineered to allow gene inactivation using antisense RNAs techniques.

Antisense RNAs can be used to specifically inhibit gene expression (see for example Eguchi et al, Annu. Rev. Biochem. 60, 631-652, 1991). Such RNA molecules can be expressed by recombinant nucleic acid molecules engineered accordingly for expression in the pig cells.

According to a further aspect of the invention, there is also provided a transgenic pig having transplantable CD40-deficient tissue or cells, preferably such comprising EC.

Animals having either one (heterozygous) or two (homozygous) inactivated CD40 genes are characterized to confirm the expected deficiency in gene expression and phenotypic effect, for example, CD40 antigen mRNA should be absent from homozygous knock-out pigs. This can be confirmed e.g. with RT PCR. Anti-CD40 antibodies can also be used in various assay to test for the presence of the CD40 epitope in an array of tissues.

1.Cloning porcine CD40 cDNA

Cloning and Sequencing.

A porcine spleen cDNA library was constructed. mRNA from porcine spleen was isolated and reverse transcribed using Timesaver cDNA synthesis kit (Pharmacia). Using ZAP Express Pre-digested Vector Kit and ZAP Express Pre-digested Gigopack III Gold Cloning Kit (Stratagene), a phagemid cDNA library was constructed. The library contained approximately 1.4×10^6 representative clones, these were amplified 100-fold. Approximately 10^6 pfu's of the amplified library were screened for pCD40 cDNA.

To provide a probe we isolated a cDNA fragment of pCD40 by RT-PCR (First strand cDNA synthesis, Pharmacia) using primers complementary to areas of sequence conserved in the human, mouse and bovine genes. (Primer A. TCGGCTTCTTCT CCAATGTGTCAT and Primer B. TCCTCCTGGGTGACCGGTTG). This cDNA fragment was sequenced and compared to human CD40 for confirmation of its identity. One round of screening of this library and 2 subsequent rounds of purification yielded four clones. The nucleotide sequence of each clone was determined by dideoxy-chain termination method (Pharmacia T7 DNA Sequencing Kit). The pCD40 cDNA was cloned into a mammalian expression vector pREP4 (Invitrogen) for expression studies. The sequences of the four putative cDNA clones were analysed using the DNASIS program (Hitachi Software).

Transfection.

Transient transfections of COS cells were performed using Superfect Transfection Kit (Qiagen). COS cells were plated at 50% confluency in six well tissue culture plates. Cells were washed in phosphate buffered saline (Gibco) before transfection and 3hrs post transfection, followed by incubation in DMEM (Gibco), 10% fetal calf serum, 2mM L-Glutamine, 200U/ml penicillin and 200ug/ml streptomycin. Transfected cells were analysed 48hrs post-transfection for cell surface expression of pCD40 and hCD40 molecules.

Cells and Cell Lines.

Jurkat E6.1, L-23 and L-52 cells were all obtained from the European collection of animal cell cultures (CAMR, Porton Down, UK). Jurkat cells were maintained in RPMI-1640

medium supplemented with 10% heat-inactivated fetal calf serum, 2mM L-Glutamine, 200U/ml penicillin and 200µg/ml Streptomycin (Sigma, Poole, UK). L-23 and L-24 cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated foetal calf serum, 2mM L-Glutamine, 200U/ml penicillin, 200µg/ml Streptomycin, 1mM Sodium Pyruvate (Sigma), and 20µM 2-mercaptoethanol (BDH). Human buffy coat was obtained from the Blood Transfusion Service, Cambridge, and heparinised pig blood was obtained from normal pigs from our own breeding colony. PBMC were prepared from both human and pig blood by density gradient centrifugation using Hypaque (Sigma).

Human CD154 binds pig CD40

A fusion protein consisting of the extracellular domain of human CD154 coupled to mouse Ig κ light chain was employed. This fusion protein is known to be biologically active in a B cell activation assay. The Jurkat cells do not bind human CD154-Ig, whereas both pig B cell lines showed a high level of binding.

While CD40 blockade has been demonstrated to be efficacious in a number of models, it remained undemonstrated whether direct signalling through the CD40 pathway is possible between porcine and human cells.The inventors have demonstrated the ability of human CD154 (hCD154) to bind pCD40 which indicates that direct signalling between a porcine graft and the immune cells of a human host is possible through this molecular pathway. From this it is reasonable to infer that CD40 signalling contributes to cellular activation during xenograft rejection and that, the inhibition or ablation of this signal could extend xenograft survival.

Human CD154 Binding Leads to EC Activation

Human Jurkat clone D1.1, which expresses CD154, or clone E6.1, which does not, were incubated for 6, 24 or 48 hours with pig EC. Jurkat D1.1 induced expression of E-selectin by 6 hrs, (peak 24 hrs) and VCAM-1 by 6 hrs (peak 24-48 hrs), whereas Jurkat E6.1 had no effect on E-selectin or VCAM-1 expression. Intriguingly, Jurkat D1.1 also induced expression of SLA class II between 6 and 24hrs (plateau at 48hrs). Again Jurkat E6.1 had no effect. Induction of E-selectin, VCAM-1 and SLA class II were all strongly inhibited when blocking mAb to CD154 was included in the cultures. The possible role of other pathways

in this activation was ruled out using the following reagents: anti-human TNFα neutralising mAb, anti-CD11a, anti-CD49d and CTLA-4-Ig. None of these affected EC activation.

These results suggest that the CD40 pathway may be involved in activation of vascular endothelium in porcine xenografts. Unlike CD40 activation of HUVEC, CD40 activation of pig aortic EC leads to up-regulation of MHC class. Since a number of cells can express CD154 including T cells, NK cells and platelets, it is possible these may contribute to EC activation. Therapies that target CD40/CD154 interactions or the signalling pathways involved reduce EC activation, and therefore improve xenograft survival.

2. Generation of Knockout Animals Through Microinjection of Eggs

a. Gene targeting constructs

The frequency of homologous recombination in embryos is improved if the gene targeting constructs are prepared with isogenic DNA. Therefore the "knock out" constructs are prepared from DNA isolated from the boar used to fertilize the oocytes used for microinjection. DNA is isolated from the tail or ear tissue, and genomic fragments from both CD40 antigen alleles of the boar encompassing the coding region are cloned using long range PCR or conventional genomic library technologies. Clones for each of the CD40 antigen alleles are identified using restriction fragment length polymorphism identification and DNA sequencing. Constructs to target both alleles are made by interrupting the coding sequence either by deletion or by inserting a heterologous DNA fragment. The constructs contain at least 8 kb of homologous DNA to promote efficient homologous recombination. Various approaches can be used to detect gene targeting events, depending on the strategies used in designing the knockout constructs. Several such approaches, and the corresponding strategies for construction of constructs, may be used as follows:

i) PCR of Genomic DNA:

Homologous DNA on one side of the interrupting DNA fragment is constructed to be less than 1 kb, allowing PCR amplification of a short diagnostic fragment. (Amplification of small fragments generally is relatively efficient).

ii) Reverse Transcription PCR:

A deletion of about 100 bp within the coding region is made, allowing synthesis of a shortened CD40 antigen mRNA in correctly targeted cells. The shortened mRNA is detected by RT PCR, using primers that amplify a fragment encompassing the deletion site.

b. Generating embryos for microinjection

Fertilized embryos are generated as described by Nottle et al., (1993). Proc. Aust Soc for Reproductive Biol. 26, 33. The protocol involves:

i) Sperm from the boar providing DNA for the targeting construct is collected and stored frozen in liquid N_2.

ii) Superovulation of donor gilts:

Gilts are mated at the second oestrus, and aborted between days 25-40 days of gestation to synchronise the subsequent oestrus cycles. Abortion is achieved by intramuscular injection of 1 mg cloprostenol (a prostaglandin F2α analogue), followed by a second 0.5 mg injection 24 hours later. Gilts are superovulated by injection of 1000 i.u. equine chorionic gonadotrophin (eCG) or pregnant mare serum gonadotrophin at the time of the second cloprostenol injection, and a subsequent injection 72 hours later of 500 i.u. human chorionic gonadotrophin (hCG).

iii) Fertilization:

Superovulated gilts are artificially inseminated 20-30 hours after the hCG injection, followed by a second insemination 2-4 hours later, with semen from the boar that provided DNA for the targeting construct.

iv) Embryo collection:

Embryos are collected surgically 50-56 hours after hCG injection prior to fusion of the pronuclei. Oviducts are flushed with 15-20 ml phosphate saline buffer containing 1 % fetal calf serum. One-cell embryos are recovered by searching oviductal flushings using low magnification microscopy.

c. Microinjection of embryos

Embryos are centrifuged at 12000 x g for 8 min to stratify the cytoplasm and allow the pronuclei to be visualised, and held in Dulbecco's Minimal Essential Medium with 25 mM Hepes and 5 mg/ml bovine serum albumin. Pronuclei are injected, using differential interference contrast optics, with 4-10 picolitres of DNA (10 ng/µl) in PBS. Gene targeting with isogenic DNA is maximized by coinjecting both allelic constructs derived from the boar into the male pronucleus.

d. Transfer of injected embryos to recipient gilts

The oestrus cycles of recipient gilts are synchronized with those of donors. The recipients are mated and aborted using the protocol described above, and injected with 500 i.u. eCG. Injected embryos are transferred surgically (20-40 per oviduct) to recipients on the same day that they are collected from donor gilts:

e. Screening for homologous recombination

Homologous recombinants can be detected by analysis of tissue from the born piglets. Screening procedures involve PCR technology, the precise strategy depending on the design of the gene targeting construct. The RT PCR approach can be more sensitive than PCR amplification of genomic DNA. The RT PCR screening strategy relies on successful transcription of the interrupted gene and relative stability of the shortened mRNA.

3. Generation of CD40 deficient animals through homologous recombination in porcine cells and nuclear transfer

a. Gene targeting constructs

The chromosomal CD40 gene in pig cells is mutated by homologous recombination with a gene targeting vector. The gene targeting vector comprises sections of DNA that are homologous to regions within or adjacent to the chromosomal CD40 gene. Ideally, these homologous sequences are identical in sequence over as large a region as is practicable. To obtain these homologous DNA sequences, total genomic DNA is purified from porcine cells or tissues. The porcine genomic DNA is used to construct a library by conventional techniques. To isolate the porcine CD40 genomic sequences, the porcine genomic library is screened using the cDNA, either whole or fragments thereof, for porcine CD40. The DNA sequence of the porcine CD40 cDNA is provided herein. The identity of isolated genomic sequences is verified using restriction enzyme mapping or DNA sequencing. DNA homologous to regions within or adjacent to the porcine CD40 gene is combined, using standard DNA manipulation techniques, with sequences which may be genes or parts of genes that facilitate, through selection or screening, the identification of cells containing the products of a gene targeting event. Gene targeting may also be performed using small oligonucleotides with DNA sequence homologous to the CD40 locus and derived from the

porcine CD40 cDNA or porcine CD40 genomic DNA. Typically, gene targeting vectors are designed to delete or inactivate the chromosomal CD40 gene.

b. Gene targeting in porcine cells

A CD40 gene targeting vector is used to mutate one or both alleles of the chromosomal CD40 gene in porcine cells. The CD40 gene targeting vector is introduced into porcine cells that are in culture in the laboratory using any of a range of DNA transfection techniques that are standard practise. These techniques include, but are not limited to, Calcium Phosphate transformation, electroporation, lipofection and micro-injection. Homologous recombination is a low frequency event and a variety of strategies may be used to identify porcine cells in which a CD40 locus has been modified in the desired way. These include, but are not limited to, the use of genes that provide drug resistance, usually the neomycin resistance gene, or genes that can be selected against, usually thymidine kinase. The polymerase chain reaction or other molecular techniques, such as Southern analysis, may also be used to identify cells that contain a modified CD40 locus. The cells used may be any porcine cell type or cell line that grows sufficiently well in culture to enable the homologous recombination event to be performed. The cells include porcine embryonic cells, porcine somatic cells grown from tissue obtained from a adult pig or a developing foetus or embryo, porcine primordial germ cells, porcine embryonic germ cells and porcine foetal fibroblasts. Porcine cells containing a mutation at one or both CD40 alleles are expanded in culture using standard techniques.

c. Nuclear transfer

Porcine cells having the desired genetic modification of the CD40 gene are used as donors for nuclear transfer. Porcine cells are fused with enucleated porcine oocytes. A micro-manipulation needle is used to introduce the porcine cell of the desired genotype under the zona pellucida of the enucleated oocyte. Fusion of the introduced cell with the oocyte is induced, usually by an electric pulse. This results in a porcine oocyte that contains the nucleus and the genetic material, including the mutated CD40 gene, of the chosen donor cell. The porcine oocyte containing the mutated CD40 gene is cultured for a period typically, but not necessarily, of four and a half hours before the oocyte is activated. Activation may be achieved chemically, for example using ionomycin. Alternative methods may be used to

activate the oocyte. The oocyte may then be implanted, immediately or following culture and further manipulation, in to the uterus of a sow. Implanted oocytes may develop to term to produce neonatal pigs that have the specific CD40 mutation that was engineered in to the cultured porcine cells. As for the cultured porcine cells, neonates may have the CD40 mutation at one or both alleles. The presence of the CD40 mutation in pigs may be demonstrated using any of a range of standard molecular techniques including, but not limited to, Southern analysis, PCR, DNA sequencing, or RFLP analyses. By standard animal breeding techniques pigs may be obtained which are heterozygous or homozygous for the CD40 mutation. These pigs may therefore be CD40 deficient. Standard breeding may also be used to generate pigs that carry the CD40 mutation in addition to further genetic modifications. These further genetic modifications include transgenes or other mutations generated by gene targeting or homologous recombination.

What is claimed is:

1. A method of ameliorating cellular graft rejection in a recipient mammal receiving a xenograft comprising using CD40-deficient donor mammal cells, tissues or organs as xenograft.

2. A method of ameliorating cellular graft rejection in a recipient mammal receiving a xenograft comprising harvesting cells, tissues or organs from a CD40-deficient donor mammal and transplanting said CD40-deficient cells, tissues or organs into the recipient mammal.

3. A porcine sample comprising cells which are genetically modified such that cell surface expression of CD40 antigen is reduced.

4. A porcine sample according to claim 3 wherein said cells are genetically modified to inactivate a gene encoding CD40 antigen.

5. A porcine sample according to claim 3 wherein said genetic modification includes insertion of antisense RNA interfering with CD40 expression.

6. A pig comprising cells having a gene encoding CD40 antigen inactivated.

6a. A pig comprising cells having a gene encoding CD40 antigen inactivated in addition to one or more further genetic modifications, gene targeted alleles or transgenes. For example, a pig comprising an inactivated CD40 gene and a transgene expressing human Decay Accelerating Factor (hDAF).

7. A DNA construct useful for inactivating a porcine gene encoding CD40 antigen by insertion of a desired DNA sequence into an insertion site of said gene, comprising said desired DNA sequence flanked by first and second homology sequences, said first and second homology sequences being respectively sufficiently homologous to first and second genomic sequences flanking said insertion site to allow for homologous recombination of

said DNA construct with said procine gene encoding CD40 antigen when said DNA construct is introduced into a porcine cell having a CD40 antigen gene.

8. A method for generating a pig totipotent cell having at least one inactivated allele of the gene encoding CD40 antigen, said pig totipotent cell being derived from a pig species having a functional gene encoding CD40 antigen, comprising:
a. providing a plurality of cells characterized as totipotent cells of said pig species;
b. introducing into said totipotent cells a nucleic acid construct effective for inactivating the gene encoding CD40 antigen by insertion of a desired DNA sequence into an insertion site of said gene through homologous recombination; and
c. identifying a totipotent cell having at least one inactivated allele of the CD40 antigen gene.

9. A method for generating a pig lacking a functional gene encoding CD40 antigen, comprising:
a. providing a pig toipotent cell having at least one inactivated allele of the gene encoding CD40 antigen, said totipotent cell being derived from a pig species having a functional gene encoding CD40 antigen;
b. manipulating said totipotent cell such that mitotic descendants of said cell constitute all or part of a developing embryo;
c. recovering a neonate derived from said embryo; and
d. raising and breeding said neonate to obtain a pig homozygous for said inactivated CD40 antigen allele.

10. A nucleic acid encoding the protein of Fig 1 fragments of said nucleic acid useful for downregulating cell surface expression of a cell surface CD40 antigen.

11. The nucleic acid of claim 10 wherein said nucleic acid is a cDNA.

FIG 1

Porcine CD40 cDNA

gcctcgcc'ATGGTTCGTCTGCCTCTGAAGTGTCTCCTCTGGGGCTGCTTTTTGACCGCCGT
CCACCCAGAACCACCCACTTCATGCAAAGAAAACCAATACCCAACAAACAGCCGGTGC
TGTAATTTGTGCCCGCCAGGACAGAAACTGGTGAACCACTGCACAGAGGTCACTGAAA
CAGAATGCCTTCCTTGCAGTTCCAGCGAATTCCTAGCCACCTGGAATAGAGAGAAACAC
TGTCATCAGCACAAATACTGCGACCCCAACCTAGGTCTCCAGGTCCAGAGGGAGGGCA
CCTCGAAAACAGACACCACTTGTGTGTGCAGTGAAGGCCATCACTGTACCAACAGCGC
CTGTGAAAGTTGCACCTTGCACAGCTTGTGCTTCCCTGGCCTCGGGGTCAAGCAGATG
GCGACAGAGGTTTCTGACACTATCTGTGAACCCTGCCCAGTTGGCTTCTTCTCCAATGT
ATCATCTGCTTCAGAAAAGTGTCAGCCTTGGACAAGCTGCGAGAGCAAAGGCCTGGTG
GAACAACGTGCGGGGACTAACAAGACCGATGTTGTCTGTGGTTTCCAGAGTCGGATGA
GAGCCCTGGTGGTTATCCCCATCACGCTGGGGATCCTGTTTGCCGTCCTGTTGGTATTT
CTCTGTATCAGAAAGGTGACCAAGGAGCAGGAGACTAAGGCCCTGCACCCTAAGACTG
AAAGGCAGGATCCCGTGGAGACGATTGATCTGGAGGATTTTCCCGACTCCACCGCTCC
GGTGCAGGAGACCTTACATTGGTGCCAGCCCGTCACCCAGGAGGATGGCAAAGAGAG
CCGCATCTCCGTGCAGGAGCGAGAGTGA

INTERNATIONAL SEARCH REPORT

Inten nal Application No
PCT/EP 99/10332

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 A61K48/00 C12N5/10 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N A61K A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	VANHOVE B ET AL : "Genetic Engineering in the pig. Gene knockout and alternative techniques" ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, vol. 862, 30 December 1998 (1998-12-30), pages 28-36, XP000906935 the whole document	1-9
X	-& Meeting paper Conference on Xenotransplantation: Scientific Frontiers and public Policy New York USA March 18-20, 1998 XP002137555	1-9

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 May 2000	25/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Cupido, M

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INTERNATIONAL SEARCH REPORT

Inten nal Application No
PCT/EP 99/10332

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X,P	WEST KA ET AL: "Cloning of the porcine costimulatory molecule CD40" TRANSPLANTATION PROCEEDINGS, vol. 31, no. 1/2, February 1999 (1999-02) - March 1999 (1999-03), pages 928-929, XP000906928 the whole document	10,11
X	-& Meeting paper from XVIIth World Congress of the Transplantation Society; Montreal, Quebec, Canada; July 12-17, 1998 XP002137556	10,11
A	WO 97 34633 A (SQUIBB BRISTOL MYERS CO) 25 September 1997 (1997-09-25) the whole document	1,2
A	ELWOOD ET ET AL: "Prolonged acceptance of concordant and discordant xenografts with combined CD40 and CD28 pathway blockade" TRANSPLANTATION, vol. 65, no. 11, 15 June 1998 (1998-06-15), pages 1422-1428, XP000906866 page 1427, right-hand column	1,2

3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 99/10332

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claimS 1 and 2 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ...nal Application No
PCT/EP 99/10332

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9734633 A	25-09-1997	AU 710998 B	07-10-1999
		AU 2331097 A	10-10-1997
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		EP 0892643 A	27-01-1999
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